Retention of Cucurbit Yellow Vine Disease Bacterium *Serratia marcescens* Through Transstadial Molt of Vector *Anasa tristis* (Hemiptera: Coreidae)

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ABSTRACT Retention or loss of transmissibility after molting was tested for adult and nymphal squash bug, *Anasa tristis* (De Geer), a natural vector of the plant pathogen *Serratia marcescens* Bizio, the causal agent of cucurbit yellow vine disease. Squash bug adults and nymphs fed from bacteria-infiltrated squash cubes were caged on squash test plants and transferred weekly to new plants for eight consecutive weeks. Twelve percent of the bugs that acquired as adults transmitted the bacterium to at least one of the test plants; 75% of these transmitters inoculated more than one plant. Transmission to plants occurred as late as 3 to 8 weeks postacquisition. Ten percent of squash bugs that fed on *S. marcescens* as fifth instars inoculated plants after molting to the adult stage; 77% of these transmitters inoculated more than one plant. Two insects that fed on *S. marcescens* as third instars inoculated squash plants. When examined by scanning electron microscopy, the foregut cibaria of transmitting insects were free of bacteria-like structures. The ability of *A. tristis* to transmit *S. marcescens* after molting to the adult stage suggests that the hemocoel acts as the site of retention of transmissible bacteria.

KEY WORDS Heteroptera, transmission, propagative bacteria, squash bug

CUCURBIT YELLOW VINE DISEASE (CYVD) is caused by the phloem-colonizing bacterium *Serratia marcescens* Bizio (Bruton et al. 2004). First reported in 1988, it was initially described in melon plants in central Texas and southern Oklahoma, but it is now known to occur in most commercially important cucurbit species throughout the midwestern and eastern United States. The squash bug, *Anasa tristis* (De Geer), a serious pest of cucurbits throughout the United States (Bextine et al. 2001, Pair et al. 2004), was identified as a field vector. *A. tristis* transmission of *S. marcescens* is unusual because 1) there are few examples of heteropteran-transmitted plant pathogens, 2) *S. marcescens* is a known insect pathogen, and 3) the majority of phloem-inhabiting plant-pathogenic bacteria and viruses are transmitted by Homoptera.

The CYVD strain of *S. marcescens* was recently shown to overwinter in adult squash bug vectors with subsequent transmission to spring-planted cucurbits (Pair et al. 2004). The relationship between the bacterium and *A. tristis* is unknown, but clues were provided by early transmission experiments (Bextine 2001). After feeding upon bacteria-infiltrated squash fruit cubes, adult squash bugs transmitted *S. marcescens* intermittently to squash plants during a 21-d testing period. The shortest time from acquisition to inoculation was that of a single adult transmitting 1–2 d after acquisition (Bextine 2001). The remainder of the transmitting insects inoculated plants after 4–6 d. It was concluded that the short latent period and the intermittent transmission patterns were more consistent with a noncirculative mode of transmission that was initially described by Purcell and Finlay (1979). A critical experiment demonstrating transmission after molting had not yet been completed for the *Anasa–Serratia* system. Here, we report transmission of *S. marcescens* by *A. tristis* adults that acquired the bacterium as nymphs, thus demonstrating transstadial retention of the bacterium. These data support a circulative route through the body of the vector before subsequent inoculation of plants can occur.

Materials and Methods

Bacteria. CYVD-causing bacteria were identified previously as *S. marcescens* by 16s rRNA and by *gro*E gene sequencing (Rascoe et al. 2003) and DNA–DNA hybridization (Zhang et al. 2003). Two cucurbit isolates, WO1 and ZO1, were first cultured from infected watermelon and zucchini squash plants, respectively, triply cloned, and stored in 30% glycerol at −80°C. WO1 and ZO1 were shown to be almost identical by genetic analysis (Zhang et al. 2003). Bacteria for all transmission experiments were thawed, grown on nutrient agar (Difco, Detroit, MI) plates, and suspended

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in sterile water to a titer of 10^8 cells/ml for immediate use. Bacteria for mortality experiments were suspended in sterile 0.1 M phosphate-buffered saline (PBS), pH 7.4.

Squash Bugs. *A. tristis* colonies were originally established from populations found in commercially grown cucurbit fields in Oklahoma. Insects were reared in 25 by 50 by 45-cm nylon-covered cages containing healthy 'Lemon Drop' squash plants supplemented with zucchini or yellow squash fruit purchased commercially. Voucher specimens were deposited in the K. C. Emerson Insect Museum, Oklahoma State University.

Bacterial Infiltration of Acquisition Cubes. Acorn squash fruit that was surface sterilized with 70% EtOH and 10% sodium hypochlorite was sliced, and the internal flesh was cubed aseptically. Cubes (8 mm^3) were placed in a sterile single vacuum port Erlenmeyer flask with the port connected via rubber tubing to a vacuum source. A suspension of *S. marcescens* WO1 or sterile water was added to the flask to a depth that did not completely cover the cubes. The flask was sealed with a rubber stopper, and a vacuum applied until air bubbles arose from the cubes (≈15 s). Then, the vacuum was released, allowing the bacterial suspension to enter the intercellular spaces. This process was repeated three times. Cubes were then wrapped tightly in stretched Parafilm and placed in a sterile petri dish until use.

Squash Bug Transmission of *S. marcescens*. Bacteria- or water-infiltrated squash cubes were placed inside 60-mm petri dishes (Fisherbrand, Fisher, Pittsburgh, PA). Insects were sorted into three developmental stages: adults, fifth instars, and third instars. Insects were given a 48-h acquisition access period (AAP) in the dishes and then transferred to two- to four-leaf squash plants (one insect per plant) enclosed in plastic tube cages (21 by 5.5 cm in diameter) for 7-d inoculation access periods (IAPs). Insects were transferred to new plants weekly for 8 wk. Test plants were placed in a greenhouse and held 4 wk for symptom expression and scored as positive if phloem discoloration characteristic of *S. marcescens* infection developed in the crown tissue (Bruton et al. 2004). This experiment was repeated six times over 2 yr.

Pathogenicity of *S. marcescens* to *A. tristis*. Adult insects were injected with *S. marcescens* ZO1 cells suspended in 0.1 M PBS, pH 7.0. A volume containing 0, 75, 150, or 300 cells (determined by direct cell count) was injected through the intersegmental membrane of the mesothoracic coxa by using a heat-drawn glass needle. Sterile PBS was injected into control insects. Injected insects were placed in petri dishes containing Parafilm-wrapped acorn squash cubes. Cubes were changed every 3–4 d for 10 d. Ten to 15 insects were injected for each treatment. The experiment was repeated three times. Mortality was analyzed using the GLM and LSMeans procedures (SAS Institute 2001).

Scanning Electron Microscopy of *A. tristis* Foregut. Internal regions of the foregut were examined for the presence of bacteria adhering to the cuticle. Heads of *A. tristis* adults that had been confirmed to be transmitters of *S. marcescens* to squash plants were fixed in 4% paraformadehyde, 0.2% glutaraldehyde for 48 h. Heads were dehydrated in a 30 to 100% ethanol series and critically point dried (Emscope model 750, Electron Microscopy Sciences, Hatfield, PA). Dried heads were cut sagittally with a razor blade to expose the interior of the ciborium and then coated for 2 min with gold/palladium by using a Balzers MED 010 sputtercoater. The precibarial region of the foregut was not exposed using this method. Heads were examined with a JEOL model JSM 6400 scanning electron microscope operated at 10 kV. Three transmitters and two healthy control insects were examined.

Table 1. Number of plants with symptoms of *S. marcescens* infection 5 wk after being fed on by *A. tristis* that had previously fed on bacteria-infiltrated cubes as adults, fifth instars, or as third instar

Replication	1	2	3	4	5	6	Total
Control adults	0/10	0/6	0/1	0/6	0/2	NT	0/25
WO1-fed adults	2/10	2/6	0/2	0/6	3/30	1/12	8/66
Control 5th instars	0/5	0/10	0/8	0/10	0/5	0/3	0/41
WO1-fed 5th instars	2/7	1/15	3/9	1/15	1/15	1/21	9/82
Control 3rd instars	0/6	0/10	0/15	0/9	0/5	0/4	0/49
WO1-fed 3rd instars	0/10	0/15	0/13	0/13	0/15	2/27	2/93

Each proportion = no. positive/no. exposed. NT, not tested.

Results

Adult Transmission of *S. marcescens* to Plants. Eight of the 66 squash bugs that were exposed in the adult stage to *S. marcescens* transmitted the bacterium to at least one squash plant. There was at least one transmitting adult in four of the six replications (Table 1). Of the adults that transmitted *S. marcescens*, 66% transmitted more than once in the eight-plant series over 8 wk (Table 2). A single adult transmitted during the first week postacquisition, and three insects transmitted during the second week. None of the 25 control insects transmitted *S. marcescens* to test plants.

Nymphal Acquisition of *S. marcescens* and Subsequent Transmission. Nine of the 82 bugs that fed on bacteria-infiltrated cubes as fifth instars transmitted *S. marcescens;* eight of these bugs inoculated plants after molting to the adult stage (Table 2). There was at least one transmitting bug in each of the six replications. Two of the nine transmitting individuals transmitted only once; the other seven bugs transmitted intermittently for 8 wk. None of the 41 control bugs that fed from water-infiltrated cubes as fifth instars transmitted *S. marcescens* to test plants. Two insects that inoculated plants after feeding on bacteria-infiltrated cubes as third instars molted to the fourth instar during the first week and to the fifth instar and adult stages by the end of the second week. Each of these insects transmitted only once during the 8-wk testing period, one transmitting during the first week and the other transmitting during the second week. None of the 49 control third instars transmitted *S. marcescens* to plants within the 8-wk testing period.

Table 2. Weekly transmission of *S. marcescens* to squash plants by *A. tristis* that fed on bacteria-infiltrated cubes as adults (A), fifth instar (N5), or third instars (N3)

SB	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8
A 1-6	−	−	+	+	+	+	pd	+
A 2-1	−	+	+	−	−	+	+	+
A 1-9	−	−	−	−	−	+	−	−
A 2-3	−	−	−	−	−	−	+	−
A 5-3	−	−	−	−	−	+	+	+
A 5-13	+	+	+	bd				
A 5-18	−	+	−	+	+	+	+	bd
A 6-8	−	−	+	+	+	pd	−	pd
N5 1-2	+	+	pd	+	pd	−	pd	pd
N5 1-3	−	+	−	+	+	+	+	−
N5 2-11	+	+	−	−	−	−	−	−
N5 3-8	−	+	−	−	+	+	−	+
N5 3-11	+	−	pd	−	−	−	−	−
N5 3-6	−	−	−	−	−	−	−	+
N5 4-5	−	−	−	−	−	+	−	+
N5 5-3	−	pd	−	−	+	−	+	+
N5 6-1	+	−	−	−	+	+	−	−
N3 6-4	−	+	−	−	−	−	−	−
N3 6-5	+	−	−	−	−	−	−	−

Plus signs (+) indicate transmission positive plants (by symptoms). Numbers in left column refer to individual squash bugs (SB) identified by replication no. − insect no. Shaded boxes indicate a molt to the next stage occurred during that week. pd, plant died. bd, bug died.

Pathogenicity of *S. marcescens* to *A. tristis* by Injection and Feeding. There was little or no mortality within 1 to 2 d after injection of CYVD *S. marcescens* strain ZO1, regardless of the three levels tested (Fig. 1). However, mortality climbed from day 3 to at least 40% after 5 d. The highest level of mortality (75% after 10 d) was achieved with the lowest bacterial concentration injected. Mortality for insects injected with 75, 150, or 300 cells was significantly higher than for PBS-injected controls on day 11 ($F = 11.20$, $P > 0.005$). Mortality of individuals that ingested the bacteria (oral route) was assessed from the transmission experiments. Forty-one percent of all insects that were exposed to bacteria-infiltrated cubes ($n = 181$, replicates 1–5) died before the end of the 8-wk experiment compared with 27% of the control insects ($n = 118$, replicates 1–5).

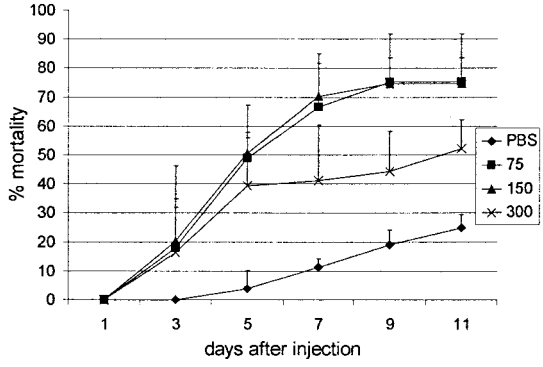

Fig. 1. Percentage of mortality of adult *A. tristis* injected with 0, 75, 150, or 300 *S. marcescens* cells suspended in PBS after 1, 3, 5, 7, 9, and 11 d. Vertical lines represent standard deviation. $n = 38$ insects (0 cells), $n = 41$ insects (75 cells), $n = 41$ insects (150 cells), and $n = 41$ insects (300 cells).

Scanning Electron Microscopy of *A. tristis* Cibaria. The foregut of *A. tristis* was difficult to access because of the thickness of the cuticle of the head capsule. It was not possible to easily remove the clypellus to reveal the epipharyngeal and hypopharyngeal regions of the foregut, which come together to form the precibarium, because the epipharyngeal wall is very thick and contiguous with the anteclypeal wall (A.W., unpublished data). Razor blade cuts bisecting the longitudinal axis exposed most but not the entire length of the cibarium. Examination of the hypopharyngeal floor of the cibarium revealed a very smooth cuticular surface with few crevices and little structural topography to interfere with plant sap flow. A central groove bisected the cibarial wall. No bacteria-like structures were found on the cibarial wall of any of the transmitting or healthy control insects.

Discussion

S. marcescens is an ubiquitous gram-negative bacterium. Strains of this species occupy many ecological niches, including that of nosocomial pathogen of humans, contaminant of hospital equipment, soil inhabitant, biocontrol agent, insect pathogen, plant endophyte, and now, phloem-inhabiting plant pathogen. How it came to be associated with the squash bug and cucurbit phloem tissues is not known. One aspect critical to the ecology of the CYVD strains of *S. marcescens* is the mechanism of its transmission by its insect vector.

Mechanisms of transmission have been well described for homopteran-transmitted plant viruses (Nault 1997) and phytopathogenic mollicutes (Fletcher et al. 1998, Purcell 1982), but much less is known about the transmission of plant-infecting walled bacteria. Bacteria transmitted by these insects

fall into two categories: noncirculative and propagative. The homopteran-transmitted noncirculative bacteria currently are represented by a single species, *Xylella fastidiosa*, the causal agent of Pierce's disease, citrus variegated chlorosis, and many other diseases of trees and shrubs (Purcell and Hopkins 1996). *X. fastidiosa*, which is restricted to the xylem of its plant hosts, is transmitted by sharpshooter leafhoppers and spittlebugs, which are specialized xylem drinkers. *X. fastidiosa* is not transmitted by noncicadelline leafhoppers, although several deltocephaline species are known to probe xylem tissues (Wayadande and Nault 1996). *X. fastidiosa* resides in the foregut of its leafhopper vectors, adhering to the cuticular lining of the food canal, precibarium, and cibarium, and forming extensive biofilms (Brlansky et al. 1983, Newman et al. 2004). Bacteria are extravasated (shed through the food canal) during the feeding process when stylets are in contact with xylem vessels. The transmission characteristics of this bacterium define a noncirculative association with the vector: *X. fastidiosa* can be transmitted immediately after acquisition. If bacteria are acquired by nymphs, transmissibility is lost after molting when the foregut cuticula is shed during ecdysis, and bacteria are retained and transmitted for life if acquired by the adult stage.

Initially, we hypothesized that *S. marcescens* shared a similar relationship with its field vector *A. tristis*, adhering to the foregut to be released through the food canal during subsequent feeding bouts. Evidence supporting this included a short period of time between the earliest possible acquisition and the latest possible inoculation (as little as 4 d) and the intermittent transmission patterns exhibited by transmitting insects (Bextine 2001). In addition, early experiments designed to test for inoculation to plants after hemocoelic injection resulted in death of injected individuals, suggesting that *S. marcescens* was pathogenic when introduced into the hemolymph (B.B., unpublished data). This also argued against a circulative route through the insect.

In this study, essentially the same results were obtained again, although the concentrations of bacteria injected (300, 150, and 75 cells per bug; Fig. 1) were lower than in the Bextine study. High mortality suggests that CYVD *S. marcescens* is pathogenic after injection into the hemocoel. We did not assess bacterial titer in injected individuals, so it is unknown whether these insects were overcome by infection, but bodies of several injected individuals examined after death had clear hemolymph and normal-looking organs and tissues, thus septicemia seems unlikely. Because so few *S. marcescens*-injected insects survived, only five plants were exposed to these insects, none of which subsequently developed yellow vine symptoms. Two scenarios could explain the lack of transmission after injection: titers in surviving insects were too low for efficient traversal into the salivary glands, or the phloem-probing behavior of the injected bugs was altered to preclude inoculation.

Determining the minimum latent period is critical for understanding how a plant pathogen interacts with its vector. Noncirculatively transmitted *X. fastidiosa* requires no latent period and is available for transmission immediately after acquisition. Short latent periods of 24 h have been documented for some circulative viruses. However, for certain propagative bacteria, such as the wall-less spiroplasmas, several days are required for cells to traverse the gut epithelia and invade the hemocoel, multiply in the hemolymph, and invade the salivary glands before the bacteria are available for introduction into a plant host via salivation (Liu et al. 1983). Our data show that most transmitting adults inoculated plants during the second week after acquisition (Table 2). The situation was different for the insects that acquired the bacteria as fifth instars, because four of the nine transmitters inoculated plants during the first week. Thus, with this group of insects, inoculation could have occurred as early as day 3 (the first day of the 7-d IAP on test plants) or as late as day 9 (the last day of the 7-d IAP).

Shorter time periods were not tested because squash bugs, unlike leafhoppers and aphids, can survive for days without food (A.W., unpublished data). Thus, feeding activity can be absent during a very short AAP such as 6, 12, or even 18 h. It is very possible that the paucity of transmissions (adult acquisition) during week 1 was due to recalcitrance of the bugs to feed on the plants or to initiate the probing of phloem or other plant tissues that may be required for *S. marcescens* inoculation. Although documented as primarily a xylem drinker (Neal 1993), *A. tristis* also probes phloem tissues (Bonjour et al. 1991), but to what extent is largely unknown. Therefore, it is impossible to know what proportion of insects that fed from bacteria-infiltrated cubes might actually inoculate plant tissue, resulting in phloem infection during a given IAP.

If *S. marcescens* were foregut-borne, then we would expect to see bacterial biofilms in the cibarium of the transmitting insects. That we did not observe bacteria adhering to the cibarium of three transmitting insects argues against the foregut as a retention site, but we were unable to examine other regions of the foregut, such as the precibarium and distal food canal of the maxillary stylets. These structures, as well as salivary gland and gut tissues of known *S. marcescens* transmitters, await examination and evaluation.

In describing the transmission parameters of the bacterium *X. fastidiosa*, Purcell and Finlay (1979) presented a definitive experiment to determine whether this bacterium would be lost during ecdysis if acquired by nymphs. When we conducted a similar experiment by using *S. marcescens* and *A. tristis* nymphs, transmission by nymphs was documented (but rare) with two insects known to be third or fourth instars at the time of inoculation. Of the 82 insects that fed on bacteria-infiltrated cubes as fifth instars, only nine transmitted the bacterium to plants. Four of these nine insects inoculated plants and also molted to the adult stage during the first week, and so it is unknown whether these four insects were nymphs or adults at the time of inoculation. Eight of the nine transmitters inoculated plants after molting to the adult stage, and in

some cases, several weeks after molting, strongly suggesting that the retention site of transmissible bacteria is within the hemocoel.

Confirmation that *S. marcescens* is propagative within its squash bug vector is consistent with the adult stage of *A. tristis* functioning as the overwintering site of the bacterium (Pair et al. 2004). However, several questions remain unanswered. How does *A. tristis* survive *S. marcescens* infection? What is the long-term survival rate of insects that acquire the pathogen by feeding? Does the bacterium become attenuated and less lethal to its squash bug host when acquired by feeding? And finally, does *A. tristis* deposit bacteria directly into the sieve tubes or is some other mechanism of phloem invasion possible? The behavior of vascular-inhabiting plant-pathogenic bacteria within their plant hosts remains poorly understood and is likely to be fundamentally different from that of vascular-inhabiting plant viruses. The answers to these questions await further study and may help us to understand how *S. marcescens* and other bacterial pathogens adapt to new ecological niches.

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